SYNOPSIS

Ph.D. in Food Science and Nutrition

ANTIOXIDANT POTENTIAL OF *Cucurbita pepo* L. (PUMPKIN) SEED EXTRACT IN THE TREATMENT OF STRESS INDUCED MALE INFERTILITY: AN IN VIVO STUDY

By

AMRUTHA B. NAIR

(17PHFNF001)

Supervisor

Dr. P.A. RAAJESWARI

Department of Food Science and Nutrition

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a) Title of the Thesis:

Antioxidant Potential of *Cucurbita Pepo* L. (Pumpkin) Seed Extract in the Treatment of Stress Induced Male Infertility: An in vivo Study

b) Brief Description of the Research Topic:

According to the latest WHO statistics, about 50–80 million people worldwide suffer from infertility (Kumar and Singh, 2015). Recent meta-analysis studies by researchers show that male factors are responsible for 20–70 percent of overall infertility cases (Masoumi *et al*., 2015; Qi *et al*., 2016). These findings are significantly broader than previously reported. Male infertility can result from testicular dysfunction, endocrinopathies, lifestyle factors, congenital anatomical factors and heavy metal exposures (Eisenberg *et al*., 2023). Heavy metals such as lead are dense elements with potential toxicity, widespread in the environment. One of the ways of toxic actions of heavy metals is related to their ability to generate Oxidative Stress (OS), causing cell damage, inflammatory processes, and apoptosis in various biological systems. Studies on the mechanisms of heavy metals associated with augmentation of OS production, as well as their complex effect on the male reproductive system, are essential in elucidating male infertility (Ilieva and Sainova, 2022). *Cucurbita pepo* L. seeds commonly known as pumpkin seeds, are a rich source of antioxidants including bioactive compounds like phenolic acids and flavonoids. They also offer numerous health benefits due to their rich nutrient profile (Batool *et al*., 2022). Considering the presence of antioxidants and therapeutic significance of *Cucurbita pepo* L. seeds, we hypothesized that an extract of *Cucurbita pepo* L. seeds could potentially contribute to mitigating oxidative stress, thereby alleviating oxidative stress induced male infertility. To test this hypothesis, the antioxidant potential of *Cucurbita pepo* L. seed extract was investigated for the treatment of oxidative stress induced male infertility using in vivo models.

c) Definition of the Problem:

Research continues to investigate the precise mechanisms linking oxidative stress to male infertility, aiming to develop targeted therapies and interventions that can effectively restore reproductive health in affected individuals. The present investigation likely addresses a gap in current knowledge regarding the specific effects of *Cucurbita pepo* L. seed extract on stress-induced male infertility. While the antioxidant properties of *Cucurbita pepo* L. seeds are established, their direct impact on reproductive health under stress conditions have not been extensively studied. By administering *Cucurbita pepo* L. seed extract, which is rich

in antioxidants, the current research intends to evaluate its effectiveness in mitigating oxidative damage and improving fertility parameters in experimental rats.

d) Objectives and Scope of Research Work:

Primary Objective

To evaluate the antioxidant potential of *Cucurbita pepo* L. seed extract in treating stress induced male infertility using in vivo models and to determine whether it can serve as a natural therapeutic source for male infertility treatment.

Specific Objectives: To

- Estimate the nutrient and antinutrient composition of *Cucurbita pepo* L. seeds
- Screen the presence of phytochemical constituents and identify the bioactive compounds in *Cucurbita pepo* L. seed extract
- Assess the *in vitro* antioxidant activity of *Cucurbita pepo* L. seed aqueous extract
- Evaluate the antioxidant potential of *Cucurbita pepo* L. seed extract in restoring the reproductive potential of stress induced infertile male rats

Scope of the Study:

There is a need for exploring the antioxidant potential of *Cucurbita pepo* L. seeds as an effective natural antioxidant source to mitigate the oxidative stress induced male infertility.

e) Methodology:

The study was carried out in five phases. In phase I, *Cucurbita pepo* L. seeds were collected from the natural habitat, an organic farm in Tudiyalur, Coimbatore district, Tamil Nadu. The collected seeds were identified, authenticated and duly certified by a taxonomist and a voucher specimen number was obtained for the same (BSI/SRC/5/23/2021/Tech/282). The seeds were dehusked and washed thoroughly under running tap water to remove any adhering debris. The seeds were shade dried for two weeks in room temperature. The dried seeds were grinded into powder and then sieved in 1mm sieve to get a fine powder. The powder was packed in an airtight container for further analyses. Nutrient analysis was carried out in triplicates using standard estimation procedures given by AOAC, 2005. The methods used were: Oven drying for moisture, incineration for ash, Kjeldahl for protein, Soxhlet extraction for fat, digestion for fibre analysis, spectrophotometry for antinutrients, minerals and vitamin and dye method for vitamin C.

In phase II, qualitative and quantitative phytochemical analyses, as well as the identification of bioactive compounds through GC-MS, were carried out. Qualitative estimation of phytochemicals was conducted using chloroform, ethyl acetate, acetone, methanol, and aqueous extracts of *Cucurbita pepo* L. seeds, following standard procedures. Carbohydrates, proteins, phenols, flavonoids, alkaloids, tannins, steroids, terpenoids, saponins, and quinones were detected qualitatively. The aqueous extract of *Cucurbita pepo* L. seeds, which showed the highest presence of phytochemicals, was selected for administration in stress-induced infertile male Wistar rats. Quantitative analysis was performed on the aqueous extract of *Cucurbita pepo* L. seeds to determine total phenolics, flavonoids, and alkaloids. The identification of bioactive compounds in the *Cucurbita pepo* L. seed extract was conducted using GC-MS.

In phase III, the aqueous extract of *Cucurbita pepo* L. seeds was examined for its *in vitro* antioxidant activity using several assays. These included the DPPH radical scavenging assay, Hydrogen Peroxide radical scavenging assay, Superoxide radical scavenging assay (SOD), and Ferric Reducing Antioxidant Power assay (FRAP), all conducted using standard procedures.

In phase IV, an acute oral toxicity study was conducted to determine the safe dosage of *Cucurbita pepo* L. seed aqueous extract for administration to experimental rats. Twelve Wistar albino female rats, 12-14 weeks old with a mean body weight (BW) of 120±35 grams, were selected for the acute oral toxicity study. The study was conducted according to OECD 423 guidelines. Animals were assigned into four groups. A single dosage administration was carried out *via* oral gavage. Control group was administered with standard diet. EG I was administered with low dosage (50 mg/kg bw), EG II with medium dosage (300 mg/kg bw) and EG III with high dosage (2000 mg/kg bw) of *Cucurbita pepo* L. seed aqueous extract. The animals were monitored for any kind of toxicity symptoms for duration of 14 days.

In phase V, the animal study was approved by the Institutional Animal Ethical Committee of Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, with the ethical number AIW:IAEC.2020:FSN:02. Thirty adult male Wistar rats, 12-14 weeks old with a mean body weight (BW) of 120±35 grams, were selected for the study. After one week of acclimatization, the male rats (n=30) were divided into five groups: Group I served as the control and was administered with standard diet; Group II (LA) was administered with lead acetate alone (30 mg/kg BW); Group III (PSE) was administered with

high dosage of *Cucurbita pepo* L. seed aqueous extract alone (1000 mg/kg BW); Group IV (LA+PSE LD) was co-treated with lead acetate (30 mg/kg BW) and a low dosage (100 mg/kg BW) of *Cucurbita pepo* L. seed aqueous extract and Group V (LA+PSE HD) was co-treated with lead acetate (30 mg/kg BW) and a high dosage (1000 mg/kg BW) of *Cucurbita pepo* L. seed aqueous extract. The dosage administration was carried out by oral gavage on 15^{th}, 30^{th} and 45^{th} day of the experimentation.

Body weight of control and experimental groups were assessed (using electronic weighing balance) on the first day and 45^{th} day of experimentation. After the experimentation, the animals were euthanized, and the vital and reproductive organs were excised out. The weights of the individual organs such as liver, brain, kidney, testes, caudal epididymis, prostate, and seminal vesicle were assessed using electronic weighing balance. The epididymis was carefully dissected out and then sperms were gently squeezed or flushed out of the epididymal tubules using a sterile buffer. The extracted sperm were collected in a sterile container. Sperm parameters such as count, motility, viability, and abnormality were analysed using staining techniques and microscopical examination. Semen pH was tested using pH paper. Serum reproductive hormonal analysis (luteinizing hormone, follicle-stimulating hormone, and testosterone) and antioxidant assays (serum superoxide dismutase, glutathione peroxidase, catalase, lipid peroxidation (malondialdehyde) and testicular tissue superoxide dismutase, catalase, glutathione peroxidase, and lipid peroxidation were carried out. Histopathology analysis was conducted by immediately fixing the dissected reproductive organs in formalin, followed by embedding in paraffin wax, and sectioning into thin pieces of 5 micrometres. Sections were stained using haematoxylin and eosin (H&E) and microscopically examined at varying magnifications to visualize tissue architecture and any pathological changes.

Statistical analysis

All data were analysed using analytics software, SPSS (Statistical Package for the Social Sciences version 21.0, SPSS Inc., Chicago, IL, USA). One way ANOVA with Dunnett's comparison test was carried out for statistical analysis and data interpretation. P values <0.001, <0.01 and <0.05 were accepted as statistically significant values.

f) Original contributions:

The salient findings of the current study are summarised below:

- *Cucurbita pepo* L. seeds contained ash (4%), moisture (4.5%), energy (559.4 kcal), total carbohydrates (11.5±0.98g), crude protein (29.8±0.72g), fat (43.8±1.02g), fibre (6.4±0.65g), calcium (48±0.25mg), iron (7.5±0.06mg), phosphorous (900±1.10 mg), potassium (450±0.98 mg), magnesium (520±0.43mg), zinc (8.2±0.12 mg), vitamin A (13.3±0.13 µg) and vitamin C (2.6±0.18mg) per 100 g of the dry weight.

- *Cucurbita pepo* L. seeds contained only negligible amount of antinutrients such as oxalates (0.16±0.05 mg), phytates (8.24±0.18 mg) and nitrates (2.11±0.04 mg) per 100 g.

- Among chloroform, acetone, ethyl acetate, methanol and aqueous extracts of *Cucurbita pepo* L. seeds, aqueous and methanol extracts contained most of the phytochemicals and a few were in appreciable levels. However, only aqueous extract showed the presence of all the phytochemicals under investigation in it. Based on the presence of phytochemicals, the aqueous extract may exhibit more therapeutic properties as compared to other extracts. Hence aqueous extract was chosen to carry out the further study.

- Quantitative estimation of phytochemical profile of *Cucurbita pepo* L. seed aqueous extract showed 32.7±0.89 mg GAE/100g of total phenolics, 16.8±0.63 mg QE/100g of total flavonoids and 11.3±1.12 mg AE/100g of alkaloids content.

- GC-MS screening on secondary metabolites in *Cucurbita pepo* L. seed extract identified seventeen prominent peaks with compounds such as 1-Tetradecanol, 1-Octadecene, n-Hexadecanoic acid (Palmitic acid), Hexadecanoic acid, ethyl ester, Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, Stigmasterol, .gamma.-Sitosterol, Squalene, Undec-10-ynoic acid, tetradecyl ester, 1,6,10,14,18,22-Tetracosahexaen-3-ol, 2,6,10,15,19,23-hexamethyl-, (all-E)-, .gamma.Tocopherol, .gamma.-Sitostenone that are considered to possess antioxidant, anti-inflammatory, anti-tumour, cancer and chemo preventive, diuretic, immunostimulant, anti-ageing, gastro and hepatoprotective, detoxifier, antimicrobial, anti-arthritic, anti-obesity, cardio and neuroprotective, anti-degenerative, prostate protective, vasodilator, antispasmodic, hypolipidemic, analgesic, anti-androgenic activities.

- The DPPH radical scavenging assay revealed that *Cucurbita pepo* L. seed aqueous extract showed a very strong scavenging activity against DPPH with percentage of inhibition (IC_{50}) value of 90.35 µg/mL, whereas for ascorbic acid, which was taken as a positive control, it was found to be 46.94 µg/mL. With respect to the exceptional IC_{50} value demonstrated by the sample when treated with DPPH, it can be deduced that the sample possess commendable antioxidant activity.

- Hydrogen peroxide (H_2O_2) scavenging assay indicated that *Cucurbita pepo* L. seed aqueous extract exhibited an inhibition with IC_{50} value of 245.81 µg/mL whereas the standard, ascorbic acid showed an inhibition of 153.33 µg/mL.

- Superoxide dismutase scavenging assay indicated an excellent scavenging activity with IC_{50} values 63.18 µg/mL and 39.15 µg/mL for the *Cucurbita pepo* L. seed aqueous extract and standard, ascorbic acid respectively.
- Ferric Reducing Antioxidant Power (FRAP) assay revealed an IC_{50} of 50 µg/mL and 25.57µg/mL for *Cucurbita pepo* L. seed aqueous extract and ascorbic acid respectively. The above findings demonstrated the *in vitro* antioxidant activity of *Cucurbita pepo* L. seed aqueous extract, which might be due to the presence of phenolic and flavonoid compounds.

- A single dosage of *Cucurbita pepo* L. seed aqueous extract ranging from 50 mg/kg body weight (low dose) to 2000 mg /kg body weight (high dose) was orally administered to female rats and were observed for 14 days. None of the rats in the experimental study showed toxicity signs in their general behaviour. Central nervous system stimulant activities such as irritability and hyperactivity exhibited immediately after supplementation of high dosage of 2000 mg/kg body weight which was subsided after 3-4 hours.

- Central Nervous System (CNS) depression activities namely hypoactivity, passivity, relaxation, and ataxia were absent in the experimental groups after supplementation of *Cucurbita pepo* L. seed aqueous extract. The Autonomous Nervous System (ANS) activities such as salivation and frequent urination after supplementation of high dosage (2000 mg/kg body weight) got diminished after 3-4 hours.

- At the end of the study (after 45 days), body weight assessment revealed a reduction in body weight of LA (lead acetate alone) group when compared to control and other

treated groups. On the other hand, PSE (*Cucurbita pepo* seed aqueous extract high dosage alone) group exhibited a weight gain similar to that of control group. A dose dependent restoring effect on the body weight was observed in LA+PSE LD and LA+PSE HD groups.

- Organ weight assessment indicated that detoxifying and excretory organs such as liver and kidney were susceptible to lead toxicity as evidenced by their weight increments in LA group. There was also a remarkable reduction in the weights of reproductive organs such as testes, seminal vesicle and prostate observed in LA group. However, there was no significant alteration to cauda epididymis and brain weights in all the treated groups. *Cucurbita pepo* seed extract when administered alone showed no significant difference in any of the organs when compared to control group whereas co-administration with lead acetate exhibited a dose dependent reversal effect on the altered organ weights.

- Sperm count results are indicative of severe lead toxicity induced oxidative stress as evidenced by a significant reduction of total number of sperms in LA group when compared to the control. On the other hand, *Cucurbita pepo* seed extract when treated alone showed a significant increment in the sperm count when compared to control. The co-treatment of seed extract with lead acetate dose dependently mitigated the lead toxicity by improving the sperm count as evidenced by LA+ PSE LD & LA+ PSE HD groups.
- Sperm motility results indicated that there was a remarkable decline of motile sperms in the lead acetate treated alone LA group which was dose dependently improved upon the administration of *C. pepo* seed extract as evidenced by LA+PSE LD & LA+ PSE HD groups. Interestingly, *C. pepo* seed extract alone treated group PSE revealed a significantly higher percentage of sperms motility when compared to the control group.
- Sperm viability results indicated that there was a notable reduction in the percentage of viable sperms in the LA group which was dose dependently improved upon the administration of *C. pepo* seed extract as evidenced by LA+PSE LD & LA+ PSE HD groups. *C. pepo* seed extract alone treated group PSE revealed a higher percentage of viable sperms compared to control group.

- Sperm abnormality results indicated a remarkable increment in the percentage of total abnormal sperms in the lead acetate alone treated group. There was dose dependent decrement observed upon the co-administration of *C. pepo* seed extract as evidenced in LA+PSE LD & LA+ PSE HD groups. *C. pepo* seed extract alone treated group PSE revealed a lower percentage of abnormal sperms when compared to control group.
- Semen pH results revealed that lead acetate alone treated group exhibited a highly significant increment in the pH making it highly alkaline in nature. On the other hand, *C. pepo* seed extract maintained a neutral pH (ideally 7.2-8) which is desirable for an effective sperm health.
- Serum antioxidant assays disclosed a highly significant reduction in the Superoxide dismutase, Glutathione peroxidase, and Catalase levels in the lead acetate alone treated LA group. LA group also markedly increased the lipid peroxidation as evidenced by high MDA level indicating the high oxidative stress induced by lead. Co-administration of *Cucurbita pepo* seed extract improved the antioxidant levels in a dose dependent manner and largely decreased the lipid peroxidation. *Cucurbita pepo* seed extract when administered alone showed a highly significant improvement in these antioxidant levels when compared to control.
- Testicular tissue antioxidant assays indicated a highly significant decline in the Superoxide dismutase, Glutathione peroxidase, and catalase levels of LA group. LA group also markedly increased the lipid peroxidation as evidenced by high Malondialdehyde MDA level. Co-administration of *Cucurbita pepo* seed extract in both low and high dosages improved these antioxidant levels besides decreasing the lipid peroxidation in a concentration dependent manner. *Cucurbita pepo* seed extract when administered alone showed a highly significant increment in these antioxidants when compared to control.
- Serum reproductive hormonal assays indicated that LA group exhibited a highly significant reduction in Follicle stimulating hormone, luteinizing hormone and testosterone levels when compared to control group. *Cucurbita pepo* seed extract treated alone PSE group retained the LH level intact similar to control group indicating no significant alteration in the hormone levels. However, co-administration of seed extract in low and high dosages concentration dependently improved the hormonal levels.
- In testis histopathology, LA group showed a decrement in the number of Leydig cells and spermatozoa with mild interstitial thickening indicating impairment in normal

spermatogenesis. PSE group showed a recovery in the tissue indicating an increased spermatozoa and normal spermatogenesis with varying stages of maturation. Co-treatment with seed extract in both low and high dosages reduced the interstitial thickening and Leydig cells were found in normal number and distribution.

- Histopathology of prostate indicated that LA group showed edematous surrounding stroma and scattered mononuclear inflammatory infiltrates whereas PSE showed normal architecture characterized by acini lined by simple columnar epithelium and basal cells luminal secretions. LA+PSE LD showed edematous stroma but there was a reduction in the mononuclear inflammatory infiltrates. However, PSE HD exhibited stroma with normal luminal secretions.
- Histopathology of caudal epididymis indicated that all groups showed normal architecture characterized by hyperplastic acini lined by simple columnar epithelium and basal cells with luminal villi projections filled with secretions.
- Histopathological examination of seminal vesicle showed degenerative changes such as glandular alterations and inflammatory infiltrates in lead acetate alone treated group (LA). *Cucurbita pepo* L. seed aqueous extract alone (PSE) showed normal glandular epithelium, tubule-alveolar glands, smooth muscle and supportive stroma. Co-treatment with *Cucurbita pepo* L. seed aqueous extract showed signs of dose dependent restoration in the histoarchitecture as evident from normal glandular epithelium, tubule-alveolar glands, smooth muscle, supportive stroma in LA+PSE LD and LA+PSE HD groups.

g) Conclusion:

Based on the findings of the present study summarized above, it may be concluded that the antioxidant potential of *Cucurbita pepo* L. seeds can be attributed to several mechanisms. Phenolic compounds and flavonoids effectively scavenge free radicals, reducing oxidative damage. Moreover, *Cucurbita pepo* L. seeds contain minerals such as zinc, which are essential for spermatogenesis and have antioxidant properties enhancing the activity of antioxidant enzymes further bolstering the body's defence against oxidative stress. Therefore, *Cucurbita pepo* L. seeds offer a multifaceted approach to combating oxidative stress-induced male infertility. Their rich content of antioxidants and minerals collectively supports sperm health by scavenging free radicals, protecting against oxidative damage, reducing inflammation, and promoting overall reproductive function. Incorporating *Cucurbita pepo* L. seeds into the diet or as a dietary supplement may provide a natural and effective strategy to

improve semen quality and enhance male fertility. *Cucurbita pepo* L. seeds hold promise as a therapeutic adjunct in the management of male infertility associated with oxidative stress.

Recommendations

1. **Safety Profile:** Assess the long-term safety and efficacy of *Cucurbita pepo* L. seed extract on general health and reproductive health.
2. **Comparison with Other Treatments:** Compare the efficacy of *Cucurbita pepo* L. seeds with other known natural and pharmaceutical treatments for male infertility.
3. **Synergistic Effects:** Explore the potential synergistic effects of *Cucurbita pepo* L. seeds with other supplements or medications.
4. **Genetic Studies:** Analyse gene expression related to fertility and antioxidant defence

h) Papers Published:

- Nair, A.B., & Raajeswari, P.A. (2023). Ameliorative Effect of *Cucurbita pepo* L. Seed Extract against Lead Induced Effects on the Serum and Testicular Oxidative Stress: An *in-vivo* Validation. *International Journal of Pharmaceutical Sciences and Drug Research*, *15*(2): 214-221. DOI: 10.25004/IJPSDR.2023.150214.

- Nair, A.B., & Raajeswari, P.A. (2024). An Exploration of the Nutritional, Phytochemical and *in vitro* Antioxidant Profile of *Cucurbita pepo* L. (Pumpkin) Seeds. *The Indian Journal of Home Science*, *36*(1):298-306.

i) References:

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